

BDNF parabrachio-amygdaloid pathway in morphine-induced analgesia



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Abstract

In addition to its neurotrophic role, brain-derived neurotrophic factor (BDNF) is involved in a wide array of functions, including anxiety and pain. The central amygdaloid nucleus (CeA) contains a high concentration of BDNF in terminals, originating from the pontine parabrachial nucleus. Since the spino-parabrachio-amygdaloid neural pathway is known to convey nociceptive information, we hypothesized a possible involvement of BDNF in supraspinal pain-related processes. To test this hypothesis, we generated localized deletion of BDNF in the parabrachial nucleus using local bilateral injections of adeno-associated viruses in adult floxed-BDNF mice. Basal thresholds of thermal and mechanical nociceptive responses were not altered by BDNF loss and no behavioural deficit was noticed in anxiety and motor tests. However, BDNF-deleted animals displayed a major decrease in the analgesic effect of morphine. In addition, intra-CeA injections of the BDNF scavenger TrkB-Fc in control mice also decreased morphine-induced analgesia. Finally, the number of c-Fos immunoreactive nuclei after acute morphine injection was decreased by 45% in the extended amygdala of BDNF-deleted animals. The absence of BDNF in the parabrachial nucleus thus altered the parabrachio-amygdaloid pathway. Overall, our study provides evidence that BDNF produced in the parabrachial nucleus modulates the functions of the parabrachio-amygdaloid pathway in opiate analgesia.

Received 5 June 2012; Reviewed 23 August 2012; Revised 30 November 2012; Accepted 27 December 2012;
First published online 21 February 2013

Key words: Brain-derived neurotrophic factor, extended amygdala, morphine, pain, parabrachio-amygdaloid pathway.

Introduction

Many aspects of the emotional, endocrine and autonomic components of pain are mediated by the spino-parabrachio-amygdaloid pathway, which sends nociceptive information to the central extended amygdala (EAC) via the parabrachial nucleus (PB) (Bernard et al., 1996). The EAC is a continuum of basal forebrain structures, stretching from the central nucleus of the amygdala (CeA) caudally to the lateral bed nucleus of the stria terminalis (BSTL) rostrally (Alheid et al., 1995; Cassell et al., 1999). In the pain context, the EAC on the one hand elaborates the affective response to pain and on the other exacerbates, facilitates or inhibits reactivity to pain, by controlling affective states (Neugebauer et al.,

2009). As such, the EAC and the neural projection transferring nociceptive signals to its components play an important role in both endogenous and exogenous analgesia, among which opioid-related analgesia.

The CeA is necessary for the expression of the morphine analgesic properties as evidenced by lesion studies in rodents and primates (Manning and Mayer, 1995; Manning et al., 2001) and morphine suppresses electrophysiological responses to nociceptive stimulus in CeA-projecting PB neurons and in CeA neurons (Huang et al., 1993a, b). The EAC is enriched with a wide range of neuropeptides, most of which are released from terminals originating from the PB (Yamano et al., 1988). One of them, calcitonin gene-related peptide (CGRP), exerts naloxone-dependent analgesic properties when injected directly in the CeA (Xu et al., 2003). In our study, we are specifically interested in brain-derived neurotrophic factor (BDNF), a neuropeptide present in axonal terminals in the EAC originating from the PB (Conner et al., 1997). In the EAC, BDNF positive terminals form pericellular

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baskets, similar to those described for CGRP (Yamano et al., 1988; Conner et al., 1997; Agassandian et al., 2006), and co-localization of CGRP and BDNF in the same terminals within CeA has been described in rats and mice (Salio et al., 2007). The BDNF role in neural plasticity is clearly established in drug addiction (Graham et al., 2007; Ghitza et al., 2010) and nociception (Pezet et al., 2002; Merighi et al., 2008). A recent study demonstrated the role of mesolimbic BDNF in the rewarding properties of morphine (Koo et al., 2012). Regarding pain studies, while most of the literature on BDNF and pain focuses on the spinal cord, the supraspinal role of BDNF remains unclear (Merighi et al., 2008). Considering the well-described role of the parabrachio-amygdaloid pathway in nociception, we formulated the hypothesis that BDNF could modulate pain mechanisms in this pathway.

In this study, we investigated the role of PB BDNF in morphine-induced analgesia using viral-mediated gene deletion in floxed-BDNF mice (Adachi et al., 2008). We first defined the parabrachio-amygdaloid pathway in mice using a tract-tracing approach. Following anxiety and motor skills assessment, we studied baseline pain threshold and morphine-induced analgesia. Morphine-induced analgesia was also tested after CeA injection of the BDNF scavenger TrkB-Fc. We then examined the impact of PB BDNF deletion on morphine-induced c-Fos expression in the EAC and in the basolateral nucleus of the amygdala (BLA). Our results reveal a critical role of BDNF in the parabrachio-amygdaloid pathway in morphine-induced analgesia.

Method

Animals

Experiments were performed using male transgenic mice homozygous for a floxed allele (exon 5) encoding the BDNF gene, with a C57BL/6J background (Rios et al., 2001). Mice were housed four to five per cage in a colony maintained at a constant temperature (23 °C) with a 12 h light/dark cycle (lights on 07:00 hours) and *ad libitum* food and water. The facilities are legally registered for animal experimentation (Animal House Agreement B67-482-1/C67-482-1) and scientists in charge of the experiments possess the certificate authorizing experimentation on living animals, delivered by the governmental veterinary office.

Surgeries

Mice were anaesthetized using either a 2.5% Avertin solution or Ketamine-Xylazine (90–10 mg/kg respectively). Surgeries were done according to a standard protocol, using glass micropipettes for tracer injections (Sarhan et al., 2005) and Hamilton syringes for viral injections (Hommel et al., 2003). The following coordinates from Bregma were used for the PB to target the external lateral portion: 14° anteroposterior (AP) angle, –4.1 mm

AP, 1.4 mm mediolateral (ML), –4 mm dorsoventral (DV). CeA targeting was –1.4 mm AP, 2.9 mm ML and –3.9 to –4.1 mm DV from dura (Franklin and Paxinos, 2008). For intra-CeA injections of TrkB-Fc, stainless 26-gauge single guide cannulas were bilaterally implanted 500 µm above the CeA. They were affixed to the skull with dental cement. A 33-gauge dummy cannula was inserted in each guide to avoid clogging and mice were allowed to recover 7–8 d before testing.

Tract tracing

Tracers were iontophoretically injected. The anterograde tracer biotin dextran amine (BDA; MW 10 000; Molecular Probes, USA; 2% in K-acetate 0.5 M) was injected in the PB using a glass micropipette broken at 10 µm tip diameter with a Midgard constant current source (+1 µA, 7 s on/off, 15 min). The retrograde tracer hydroxystilbamidine methanesulfonate (Fluoro-Gold®, FG, Fluorochrome, USA; 2% in NaCl 0.5 M) was injected in the CeA with a glass micropipette broken at 50 µm tip diameter (+3 µA, 7 s on/off, 10 min). Six to eight days after injection, anaesthetized animals were fixed by paraformaldehyde perfusion, brains were removed, sectioned on a vibratome (Leica, Germany; 40 µm, frontal plane) and treated for BDA histochemical revelation (Sarhan et al., 2005) or analysed under the fluorescence microscope (FG injection).

Adeno-associated virus (AAV)-mediated deletion of BDNF

Local expression of either enhanced green fluorescent protein (eGFP) alone or eGFP fused to the Cre recombinase was achieved using viral-mediated gene delivery (Berton et al., 2006). The DNA constructs were cloned into an AAV-2 vector in which the genes of interest were under the control of the ubiquitous cytomegalovirus promoter. Viral production was accomplished using a triple-transfection, helper-free method and purified as described earlier (Hommel et al., 2003). A total of 0.5 µl was bilaterally injected into the PB over 5 min (0.05 µl/30 s) and the needle was removed after 10 min. The age of mice at the time of deletion ranged from 9 to 14 wk. Behavioural tests were conducted at least 3 wk (usually 4–5 wk) following AAV injections.

BDNF expression assessment

In situ hybridization was done using a 1085 bp probe based on the rat BDNF gene (accession number NM_012513). The probe was labelled with [³⁵S]UTP, purified and used at 350 pM. Cryostat (Microm, France) frontal sections (20 µm) were fixed, acetylated, dehydrated and de-fatted prior to hybridization with the probe in hybridization buffer at 54 °C overnight. Sections were then washed, fixed, treated with RNase and dehydrated, left to dry, then exposed to BioMax MR films for 6 d.

Behavioral scoring

Assessors were blind to group assignment. Anxiety levels were assessed with the dark–light, open-field and elevated plus-maze tests. Tests were performed during the light phase, under controlled light conditions (90 lux), videotaped and analysed using an automated video-tracking system (Ethovision, USA). These tests were conducted using the same animals with a 1 wk space between tests.

Dark–light

The dark–light test was performed in place-preference boxes (Med Associates, USA). One compartment was dark while the other larger one was lit. Animals were placed in the dark compartment for 2 min and the door between the two compartments was opened allowing the animal to freely move from one to the other for 10 min. Photoelectric beams at the door detected when the animal explored or entered the light compartment. Total time spent in lit compartment was measured.

Open-field

The open-field test was conducted over 10 min. Animals were placed in the centre of the apparatus (49 cm × 49 cm boxes). The average distance relative to the borders of the box and the total distance were measured.

Elevated plus-maze

Mice were placed in the centre of the maze, the head in the direction of a closed arm. Over a 5 min period, they were evaluated for the time spent in the open and closed arms (55 cm from the floor, 12 cm × 50 cm arms; Monteggia et al., 2007).

Rotarod

The rotarod test was performed to evaluate motor coordination. Animals were placed on immobile cylinders, which ramped up from 0 to 45 rotations/min (IITC, USA). The timer was stopped when the mouse fell off the cylinder or did a whole turn with it. This procedure was repeated three consecutive times.

Nociceptive tests

Mechanical noxious threshold was determined using the von Frey microfilaments (Bioseb, France) as described previously (Yalcin et al., 2011). Mice were placed in Plexiglas boxes (7 cm × 9 cm × 7 cm) on an elevated grid. Calibrated filaments were applied on the plantar surface of the hindpaw. The filaments were tested five times per paw in ascending forces until the paw withdrawal threshold was reached (Barrot, 2012). Noxious threshold was defined as the lower filament for which three or more withdrawals were observed. The cut-off was set at 15 g.

Thermal noxious response was determined using a hot plate (IITC). Mice were placed on a 52 °C plate, in a Plexiglas cylinder and latency to first paw licking was used to define noxious thermal threshold. The cut-off time was set at 50 s.

To evaluate opiate-induced analgesia, morphine (10 mg/kg) and saline were injected s.c. 30 min prior to testing. Mechanical and thermal responses were assessed in independent sets of mice.

Intra-CeA TrkB-Fc injection

Mice implanted with guide cannulas were tested for morphine-induced mechanical analgesia after CeA bilateral infusion of the BDNF scavenger TrkB-Fc. TrkB-Fc (0.5 µl per side; R&D Systems; 0.1 µg/µl in PBS) or PBS was delivered via a 33-gauge cannula that extended 500 µm below the end of the guide cannula. Injections were performed at a constant rate over 2.5 min (CMA 400 Syringe Pump). The cannula was left in place for 5 min before removing it. One hour later, mice received morphine (10 mg/kg s.c.). Mechanical noxious thresholds were determined 20 min before morphine injection (40 min after intra-CeA TrkB-Fc or PBS infusion) and 20 min after morphine injection (80 min after TrkB-Fc or PBS infusion).

Morphine-induced c-Fos response

Five wk following AAV injection in the PB, animals received a saline injection the day prior to the experiment for habituation to the procedure. The day of the experiment, 2 h after morphine (10 mg/kg s.c.) or saline injection, animals were anaesthetized and fixed using standard procedures (Sarhan et al., 2005). Brains were sectioned on a vibratome (40 µm frontal sections) and immunostained for c-Fos. Free-floating sections were washed in PBS and endogenous peroxidase activity was blocked (50% ethanol, 1% H₂O₂). After PBS washes, aspecific binding sites were saturated in 5% donkey serum in PBS 0.3% triton (PBS-t) and incubation in primary antibody (rabbit anti-c-Fos 1:2000, Santa Cruz Biotechnology, SC-52; 1% donkey serum; PBS-t 0.3%) was done overnight (Kaufling et al., 2009). Next, sections were washed, incubated with secondary biotinylated antibody (donkey anti-rabbit, 1:200, GE Healthcare, UK; 1% donkey serum), washed and incubated in ABC kit (1:500; ABC Elite, Vector Laboratories, USA). Diaminobenzidine revelation was performed (DAB 0.0125%, H₂O₂ 0.0009%) and after extensive washes, sections were mounted on gelatine-coated slides, air-dried and coverslipped with Eukitt (O. Kindler GmbH, Germany). c-Fos positive nuclei were counted in the BLA, the dorsal BSTL and the capsular (CeC), lateral (CeL) and medial (CeM) subdivisions of the CeA using a microscope attached to a camera Lucida. On average, four sections per animal were counted for the BSTL and 4–6 for the BLA and the CeA.

Histology

At the end of the behavioural experiments, anaesthetized animals were fixed and the brains were sectioned on a vibratome (40 μ m frontal sections). For BDNF PB deletion experiments, the PB was examined under fluorescence for eGFP expression. For intra-CeA TrkB-Fc experiments, the bilateral placement of cannulas was assessed on Cresyl Violet stained sections. Pictures were taken using an epifluorescence microscope (Leica) with a digital camera (Cool Snap, USA) or using a Nikon E80i microscope. Adobe Photoshop (Adobe, USA) was used to adjust contrast, brightness and sharpness.

Statistical analysis

Data are expressed as mean \pm S.E.M. and analyses were performed with STATISTICA 8 (Statsoft, USA). To assess effects on morphine-induced analgesia, multifactor analysis of variance was used. When appropriate, the Neuman–Keuls test was used for *post hoc* comparisons. For analysis of c-Fos induction, Student's *t* test was used for two groups' comparisons. The significance level was set at $p < 0.05$.

Results

Anatomical substrate for the parabrachial BDNF projection

Tract tracing

Anterograde tracer injections ($n=2$ for successful placement of injection site) targeting the PB (lateral PB, external part, PBel; Fig. 1i) led to terminal fibre labelling in the CeL, the CeC and the dorsal BSTL (Fig. 1ii, iii), with very few labelled regions outside of them. Moreover, retrograde tracing from the CeA ($n=2$ for successful placements) resulted in strong labelling of cell bodies concentrated into the PBel (Fig. 1iv, v). These results establish that our subsequent AAV injections targeted the PB-EAc pathway.

Local BDNF deletion

The placement and extent of the AAV injections were assessed by the presence of eGFP-positive neurons for both control (eGFP alone) and BDNF-deleted animals (eGFP-Cre fusion). eGFP labelling had to be present and concentrated in the lateral PB for the injection placement to be considered as valid, although it often also included labelling spreading into the median PB. The pontine PB was the main targeted area, but some injections displayed a larger rostro-caudal spread, thereby also including the mesencephalic PB (Fig. 2a, b, d). eGFP expressing neurons were often clustered in the external aspect of PB, namely, the external lateral, lateral crescent and external medial nuclei. Animals were included for

data analysis if the viral infection comprised the lateral PB, remained within the boundaries of the PB and was validated bilaterally.

Cellular aspects of the eGFP labelling differ whether or not it is fused to the Cre recombinase. eGFP alone, in control animals, is cytoplasmic and displays a labelling that fills the entire soma as well as its dendritic (Fig. 2c) and axonal processes. Terminal axonal labelling can be observed in brain regions receiving afferents from the injection site. Strong eGFP labelling was found in both the lateral part of the CeA (CeL and CeC) and the dorsal BSTL for all control animals (Fig. 2f–h). Moreover, the eGFP-positive terminals in these structures display the characteristic pericellular basket appearance (Fig. 2h) described for CGRP-containing terminals in the CeA and BSTL. When fused to the Cre recombinase, the eGFP labelling appears nuclear (Fig. 2e), while the number of infected cells per injection site was comparable in both control and deleted animals.

In agreement with previous reports (Berton et al., 2006; Monteggia et al., 2007; Adachi et al., 2008; Koo et al., 2012) in different brain regions, *in situ* hybridization reveals a decrease in BDNF mRNA levels in BDNF-deleted animals (Fig. 2i, j). In all animals, BDNF appears concentrated in few brain nuclei, but BDNF transcripts were highly expressed in the PBel in control animals, as opposed to AAV-eGFP-Cre injected animals.

Parabrachial BDNF mediates morphine-induced analgesia

General behavioural assessment

Following histological validation, 10 control and 10 BDNF-deleted animals were used for behavioural analyses. Prior to morphine-induced analgesia measurement, animals were assessed for their anxiety levels and motor skills. No differences were noted for anxiety levels as measured by the dark-light, elevated plus-maze and open-field tests (Fig. 3a–d). Similarly, motor coordination measured using the rotarod was not altered by BDNF deletion in the PB (Fig. 3g). Finally, no difference was observed for general locomotor activity in the open-field test (Fig. 3f).

Morphine-induced analgesia

In the hot-plate test, control and BDNF-deleted animals displayed similar responses following saline injections (Fig. 4a). Morphine-induced analgesia was present in both groups, but it dramatically decreased in PB BDNF-deleted animals (38% reduction, $p < 0.001$). Both controls and BDNF-deleted animals showed a tolerance to the analgesic properties of morphine (Fig. 4a), with the largest drop in efficacy noted for the second day of morphine injection. Following tolerance procedure, thermal nociceptive response was reassessed, with both groups

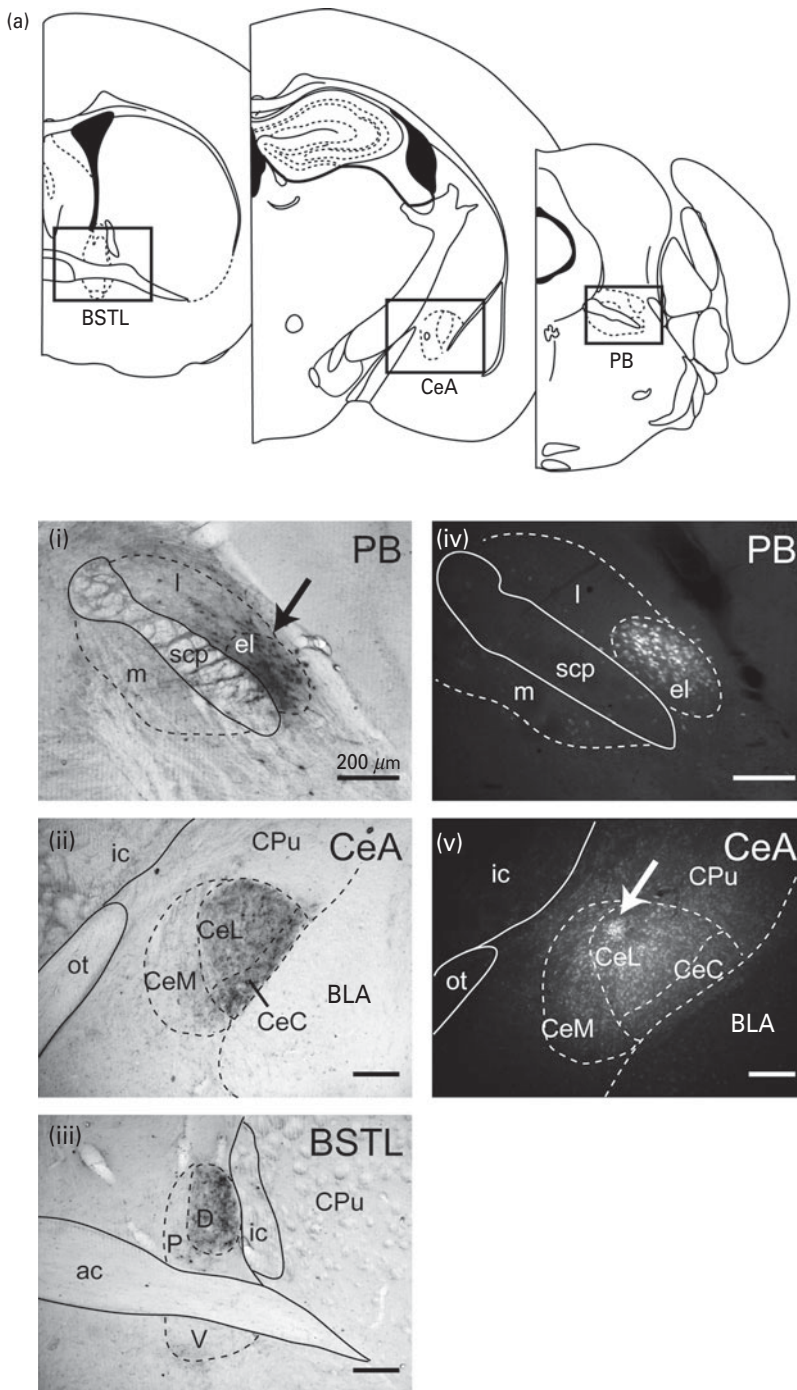


Fig. 1. Parabrachio-amygdaloid pathway in mouse. (a) Shows the structures of interest (boxed areas) on frontal brain sections drawings (adapted from Franklin and Paxinos, 2008). After deposit of biotin dextran amine in the parabrachial nucleus (PB; i, black arrow), anterogradely labelled axons are found in the lateral part of the central nucleus of the amygdala (CeA; ii) and in the dorsal part of the lateral bed nucleus of stria terminalis (BSTL; iii). Retrogradely labelled somas are found in the PB (iv) after Fluoro-gold[®] injection in the CeA (v, white arrow). Scale bars: 200 μ m. ac, anterior commissure; dorsal (D), ventral (V), posterior (P) parts; BLA, basolateral amygdala; lateral (CeL), capsular (CeC), medial (CeM) parts; CPu, caudate putamen; ic, internal capsule; ot, optical tract; lateral (l), lateral external (el), medial (m) parts; scp, superior cerebellar peduncle.

again showing similar values in response to saline injections (Fig. 4a).

Mechanical nociceptive response was evaluated in an independent experiment. Similar to thermal sensitivity,

no baseline difference was noted between control and BDNF-deleted animals (Fig. 4b), but morphine-induced analgesia was reduced in BDNF-deleted animals (58% reduction, $p < 0.001$; Fig. 4b).

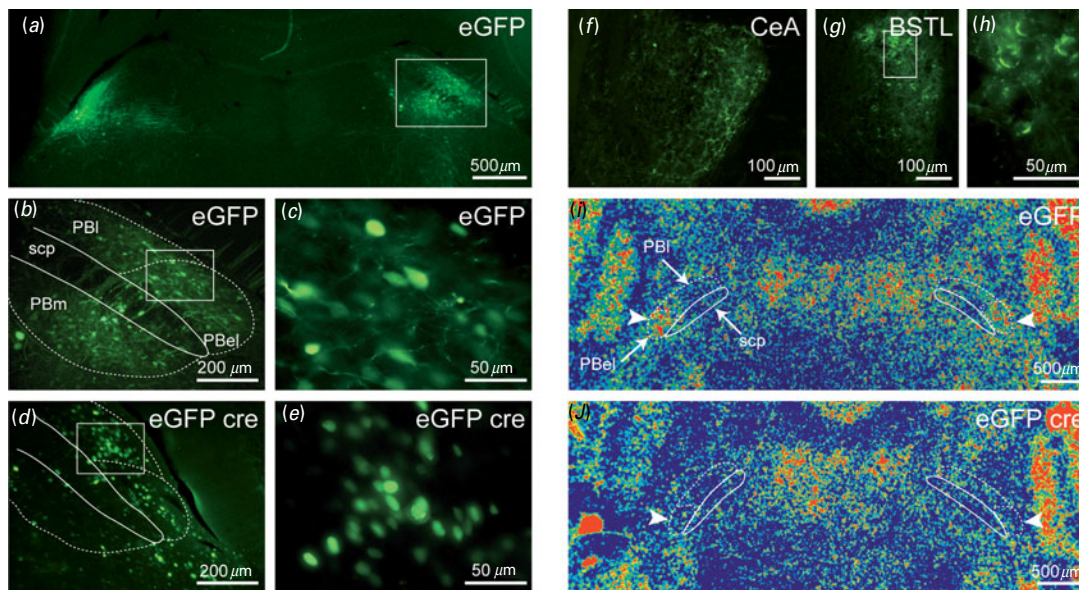


Fig. 2. Adeno-associated virus (AAV) injections. Enhanced green fluorescent protein (eGFP) expression in the parabrachial nucleus (PB) following AAV-eGFP (*a–c*) or AAV-eGFP-Cre (*d, e*) injections. Following AAV-eGFP injection in the external lateral PB (PBel), fluorescent terminals are observed in the central nucleus of the amygdala (CeA; *f*) and in the lateral bed nucleus of stria terminalis (BSTL; *g, h*) with typical perisomatic basket appearance. *In situ* hybridization, with optical density changes converted to a colour scale shows a decrease in brain-derived neurotrophic factor (BDNF) mRNA levels in the external lateral PB (white arrowheads) of AAV-eGFP-Cre injected mice (*j*) as compared to AAV-eGFP controls (*i*). Lateral (l), medial (m) parts; scp, superior cerebellar peduncle.

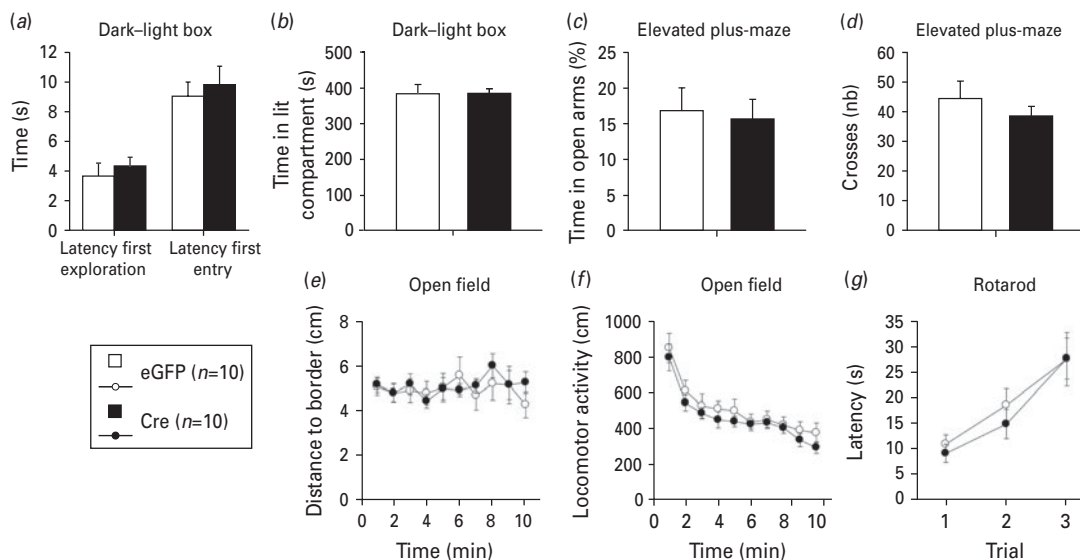


Fig. 3. Behavioural measure (\pm S.E.M.) of anxiety using the dark-light (*a, b*), the elevated plus-maze (*c, d*) and the open-field (*e, f*) tests. Locomotor activity was assessed with the open-field (*f*) and motor coordination with the rotarod test (*g*). eGFP, enhanced green fluorescent protein.

BDNF into the CeA mediates morphine-induced analgesia

Following histological validation of cannula placement, morphine-induced mechanical analgesia was analysed on nine mice with intra-CeA PBS (control mice) and six mice with intra-CeA TrkB-Fc (TrkB-Fc mice). The nociceptive thresholds before morphine injection were similar between control and TrkB-Fc mice (Fig. 4c), but a

decrease in morphine-induced analgesia was observed in TrkB-Fc mice (49% reduction, $p < 0.001$; Fig. 4c).

Parabrachial BDNF mediates morphine-induced early cellular responses

In both control and BDNF-deleted animals, morphine injection increased the number of c-Fos positive nuclei in the dorsal BSTL, the BLA and the three subdivisions of

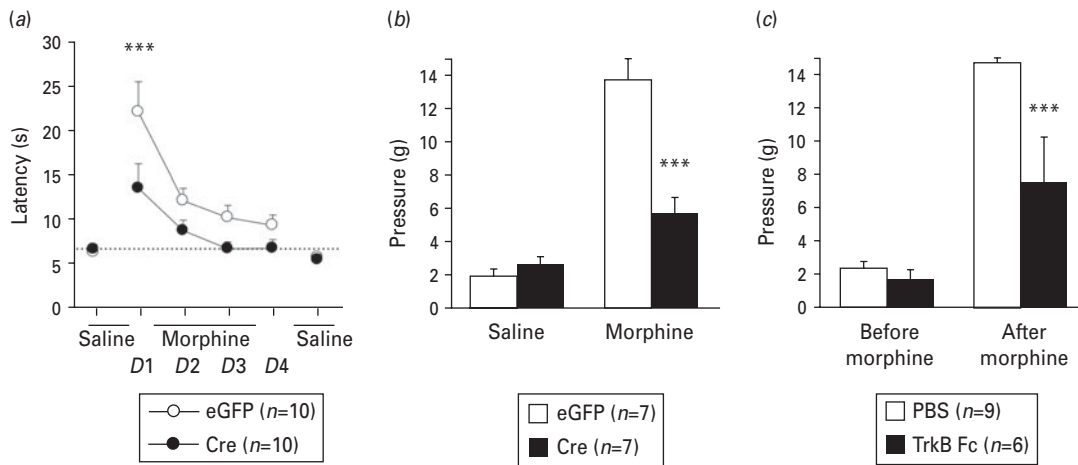


Fig. 4. Effect of brain-derived neurotrophic factor parabrachial nucleus deletion (a, b) and TrkB-Fc infusion into central nucleus of the amygdala (c) on thermal (a) and mechanical (b, c) nociceptive responses during morphine analgesia (mean \pm S.E.M.). *** $p < 0.001$. eGFP, Enhanced green fluorescent protein.

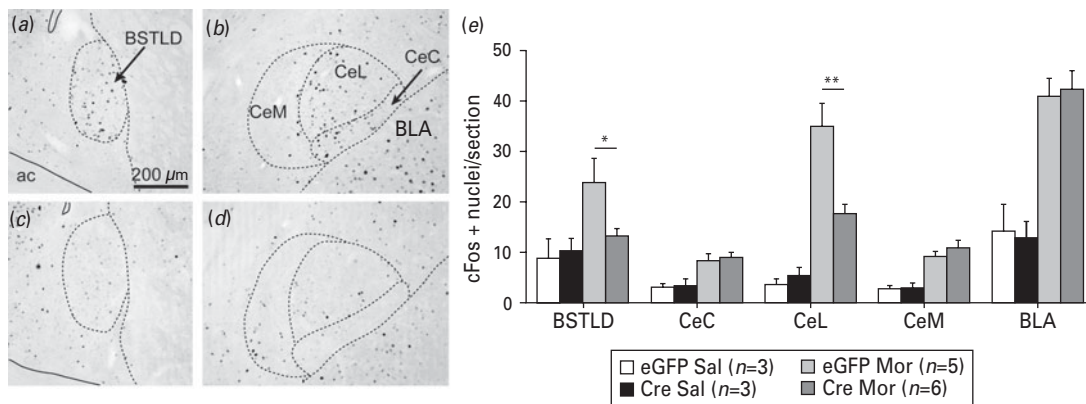


Fig. 5. Morphine-induced c-Fos in control (a, b) and brain-derived neurotrophic factor-deleted (c, d) animals in the lateral bed nucleus of stria terminalis dorsal part (BSTLD; a, c) and in the amygdala (b, d). Quantification (\pm S.E.M.) in the BSTLD, the central nucleus of the amygdala (CeA) subdivisions and the basolateral amygdala (BLA; e). * $p < 0.05$, ** $p < 0.03$. ac, Anterior commissure; lateral (CeL), capsular (CeC), medial (CeM) divisions; morphine (Mor); saline (Sal).

the CeA (Fig. 5e). In control animals, the number of c-Fos positive nuclei was at least doubled (BSTL 171%, CeC 176% and CeM 233% over saline), with the largest morphine-induced elevations being counted in the CeL (929%) and the BLA (187%; Fig. 5e). No difference between control and BDNF-deleted animals is noted for responses to saline, but these groups differ widely when comparing their response to morphine (Fig. 5a–d). More specifically, c-Fos positive nuclei were reduced by approximately 50% in both the dorsal BSTL (control *vs.* BDNF-deleted groups, $p < 0.05$) and the CeL ($p < 0.05$), while the CeC, CeM and BLA were equally responsive to morphine regardless of the PB deletion (Fig. 5e).

Discussion

We used tract-tracing techniques to visualize the mouse parabrachio-amygdaloid projection, a pharmacological

approach to block BDNF actions in the CeA and molecular tools to delete the BDNF gene in the PB. From a cellular perspective, this deletion led to a decrease in c-Fos induction in the CeL and BSTL following morphine administration. From a behavioural perspective, it resulted in decreased morphine-induced analgesia. This reveals an essential role for the BDNF parabrachio-amygdaloid pathway in opiate-induced analgesia.

Morphological and functional aspects of the spino-parabrachio-amygdaloid nociceptive pathway have been thoroughly described in rats (Bernard et al., 1996; Sarhan et al., 2005). While it is expected to be similar in mice, supporting evidence is only starting to emerge (Tokita et al., 2010). Here, both anterograde and retrograde tracing methods showed that neurons in PBel extensively project to the EAc in mice. In addition, the anterograde transport of eGFP expressed by AAV-infected neurons in the PBel led to terminal labelling in the EAc, thus

confirming the targeting of the PB-EAC pathway. It should also be noted that, in the rat, PB neurons projecting to the EAC, notably those in the PBel, do not innervate other structures except a light projection to the lateral hypothalamus and midbrain reticular formation (Sarhan et al., 2005).

BDNF immunoreactivity was described in the CeA and BSTL in rats and mice (Conner et al., 1997; Yan et al., 1997; Krause et al., 2008). In the PB, a BDNF immunoreactive plexus is described along with BDNF expressing neurons (Ceccatelli et al., 1991; Conner et al., 1997) and these neurons project to the ipsilateral EAC (Conner et al., 1997). Distinctive pericellular structures are described for the EAC BDNF terminal labelling, featuring a similar morphology to the axonal terminals originating from the PB-EAC projection (Conner et al., 1997; Sarhan et al., 2005; Agassandian et al., 2006). Locally, dense core vesicles were shown to contain simultaneously BDNF, substance P and CGRP (Salio et al., 2007). Furthermore, the BDNF receptor TrkB was post-synaptically described in the EAC (Yan et al., 1997; Agassandian et al., 2006). Behaviourally, both EAC (Davis et al., 2010) and BDNF (Rattiner et al., 2004; Monteggia et al., 2007) were implicated in anxiety-like behaviours. Indeed, BDNF global deletion leads to enhanced anxiety-related behaviour (Rios et al., 2001). However, our results suggest that the parabrachio-amygdaloid pathway may not be relevant in this context since local manipulation of BDNF in the PB is not accompanied by a change in anxiety.

The spino-parabrachio-amygdaloid pathway carries nociceptive information to the EAC, which then mediates sensorimotor, emotional and affective dimensions of pain (Neugebauer et al., 2009). This is achieved via connections with ascending and descending nociceptive systems (Oliveira and Prado, 2001; Neugebauer et al., 2009). Lesion studies demonstrated that the CeA is not critical for generating the physical response to acute pain, but it is involved in the expression of several forms of analgesia, such as conditioned- or stress-induced (Helmstetter, 1993; Helmstetter et al., 1993; Watkins et al., 1993; Manning and Mayer, 1995). The CeA inactivation or lesion also reduces morphine-induced analgesia (Manning and Mayer, 1995; Manning et al., 2001) and, while systemic morphine is expected to exert its properties on multiple relays along the pain matrix, this indicates that CeA is a required element of the anti-nociceptive circuit recruited by morphine. Moreover, morphine and β -endorphin injections into the amygdala were shown to induce analgesia through periaqueductal grey (PAG) connections (Helmstetter et al., 1993; Pavlovic and Bodnar, 1998), thus suggesting that morphine can directly act on CeA circuitry. The three opioid receptors subtypes are present in the EAC, both pre and post-synaptically (Zhu and Pan, 2005; Jaferi and Pickel, 2009; Poulin et al., 2009). In the three CeA subdivisions, neurons can be post-synaptically inhibited by μ -receptor agonists (Chieng et al., 2006), but presynaptic inhibition of glutamate or GABA release

has also been reported (Finnegan et al., 2005; Zhu and Pan, 2005). We found that acute morphine injection increased the number of neurons expressing c-Fos in the CeA, dorsal BSTL and BLA, which is consistent with previous reports (Hamlin et al., 2007). As almost all EAC neurons are GABAergic (Alheid et al., 1995; Cassell et al., 1999), and activation of μ -receptors usually results in an inhibitory outcome, the local effects of morphine would mainly be due to disinhibitory mechanisms. Thus, morphine would mimic the physiological activation of the opioid system in the CeA to promote anti-nociceptive processes normally activated during life-threatening situations. The cellular mechanisms underlying this activation are, however, poorly understood.

Part of the inhibitory circuit of the mice CeA has been recently disclosed in the context of fear conditioning (Ciocchi et al., 2010; Haubensak et al., 2010). It has been suggested that, in the CeL, GABA neurons (OFF cells) tonically inhibit CeM projection neurons responsible for fear-induced freezing. Another population of GABA CeL neurons (ON cells) can be excited by BLA and/or cortical afferents following presentation of a shock-paired tone. As a consequence, ON cells would inhibit OFF cells, thus disinhibiting CeM neurons and triggering the activation of defensive pathways in the brainstem. As hypoalgesia occurs during expression of conditioned fear, it could be suggested that a similar circuit in the CeA controls conditioned analgesia. However, while Finnegan et al. (2005) proposed that μ -receptor agonists trigger the disinhibition of CeM PAG-projecting neurons, Chieng and Christie (2009) suggest the contrary, i.e. inhibition of CeM projection neurons. Several neuropeptides such as oxytocin, neurotensin, corticotrophin-releasing factor and CGRP also induce opioid-dependent anti-nociception when injected into the CeA (Kalivas et al., 1982; Xu et al., 2003; Cui et al., 2004; Han and Yu, 2009). This would be achieved, at least for oxytocin, through the inhibition of CeM projection neurons since oxytocin activates ON cells in the lateral CeA and decreases freezing (Haubensak et al., 2010; Knobloch et al., 2012). Finally, in addition to its anti-nociceptive roles, the CeA is also involved in pro-nociceptive processes, especially during prolonged pain (Neugebauer et al., 2009). A bi-directional control of CeA output has thus to be achieved either through a single circuit with both anti- and pro-nociceptive potentialities or through the balance between two opposite circuits. Our results show that deleting BDNF from the PB-EAC pathway, or directly inhibiting BDNF actions in the CeA, reduces morphine-induced analgesia. It is thus possible that BDNF is involved in maintaining the balance in CeA circuits and in tuning the reactivity to emotionally relevant situations.

BDNF has been thoroughly studied in the context of pain modulation, but most studies focused on the spinal level (Pezet et al., 2002; Ren and Dubner, 2007; Merighi et al., 2008). BDNF involvement in supraspinal pain

mechanisms was explored in the PAG and its main downstream effector, the rostroventromedial medulla (RVM). Midbrain BDNF infusions (Guo et al., 2006) as well as intracerebroventricular administrations (Cirulli et al., 2000) appear anti-nociceptive without affecting basal nociceptive thresholds. In contrast, BDNF transmission within the RVM appears pro-nociceptive and facilitates hyperalgesia (Guo et al., 2006). Thus the BDNF pathways are part of descending pain control systems with both anti-nociceptive and pro-nociceptive roles. Interestingly, TrkB controls morphine-induced analgesia without affecting basal nociceptive responses (Lucas et al., 2003). Our results are consistent with these data, since suppressing BDNF in the PB-EAC projection results in unaltered basal pain responses, but decreases morphine-induced analgesia. From a cellular aspect, morphine increases c-Fos expression in all analysed EAC nuclei as well as in the BLA. In BDNF-deleted mice, this induction is reduced in the CeL and dorsal BSTL, but not in the BLA. Since the CeL and the dorsal BSTL are directly innervated by PB axons containing BDNF (Conner et al., 1997), it appears that cellular recruitment in the EAC in response to morphine is, at least partially, dependent on BDNF released by PB terminals.

Our results suggest that BDNF is necessary to obtain effective morphine-induced analgesia. Several mechanisms can be proposed. BDNF may act in the PB itself by modifying dendritic morphology (Horch, 2004) or by modulating the expression of neuropeptides such as CGRP in parabrachio-amygdaloid neurons. If BDNF regulates PB expression and/or release of CGRP, it would eventually impact its main anatomical target, the EAC. However, the role of CGRP in pain-related mechanisms in the CeA remains controversial, both pro-nociceptive (Han et al., 2010) and anti-nociceptive (Xu et al., 2003) actions being reported. While we cannot rule out a local action of BDNF in the PB, the behavioural effect of TrkB-Fc infusion in the CeA shows that BDNF can modulate morphine analgesia by an acute local effect in the CeA. BDNF-TrkB signalling is able to facilitate glutamatergic transmission (Guo et al., 2006; Ren and Dubner, 2007; Merighi et al., 2008) and also leads to the rapid internalization of GABA_A receptors in the amygdala, allowing for transient hyperexcitability (Mou et al., 2011). In the CeA, BDNF is present in pericellular pre-synaptic terminals containing glutamate (Agassandian et al., 2006; Delaney et al., 2007). Post-synaptically, both BDNF and TrkB immunoreactivities are detected on post-synaptic densities of asymmetric synapses facing BDNF-positive axons, presumably from PB origin, but also BDNF-negative axons from BLA or cortical origin (Agassandian et al., 2006). PB-derived BDNF in the CeA may thus modulate excitatory synapses. In a given physiological situation, this would allow selecting a specific circuit by targeted disinhibition via the opioid system and enhanced excitation via the BDNF system. During morphine analgesia, μ -receptors in the CeL could thus trigger the silencing of a GABA

population, thus disinhibiting a second population that could be activated by BDNF-potentiated excitatory afferents. The absence or blockade of BDNF in our experiments would reduce this excitation, as suggested by the decreased c-Fos recruitment in the CeL, and consequently reduce the positive influence of CeA on anti-nociceptive processes. Interestingly, the CeC has often been associated with pro-nociception while the CeL has been more associated with anti-nociception (Hamlin et al., 2007; Neugebauer et al., 2009). It is, however, too speculative to further develop a potential circuit for BDNF action in the CeA. First, we do not know if PB inputs preferentially target ON or OFF cells or another group. The PB in the rat indeed projects to CeL and CeC via different neuronal populations (Sarhan et al., 2005) and the distribution of ON and OFF cells in the CeA has not been described with enough precision to differentiate between CeL and CeC. Second, the correlation between the CeA distribution of μ -receptors and their cellular and behavioural effects is only partial and controversial. Third, whereas morphine can directly act on μ -receptors in the CeA, the overall changes in CeA activity, including those involving BDNF, can also be due to μ -receptors in CeA-projecting structures, such as the intercalated cell masses, which provide an inhibitory input to the CeA (Palomares-Castillo et al., 2012). Dissecting the mechanisms of BDNF action in the CeA will thus need additional anatomical and functional studies.

The potential role of BDNF in the BSTL remains elusive. We show that BDNF deletion in the PB-EAC pathway leads to decreased morphine-induced c-Fos activation in the dorsal BSTL, similar to what was observed in the CeL. The CeA and the BSTL share similar cytoarchitectural, neurochemical and hodological features and are strongly interconnected (Alheid et al., 1995; Cassell et al., 1999). BDNF innervation in the BSTL originates in the PB (Conner et al., 1997) and PB-BSTL axons arise as collaterals of PB neurons innervating the CeA (Sarhan et al., 2005). However, our TrkB-Fc experiment shows that blocking BDNF signalling in CeA is sufficient to decrease morphine-induced analgesia. Indeed, while the role of BSTL in pain is less studied than the CeA, it may be mainly related to affective and neuroendocrine dimensions (Deyama et al., 2007), with little or no evidence of an involvement in nociceptive sensitivity. Thus, BDNF in the BSTL may be involved in a circuit similar to the CeA, but with a preferential impact on the affective aspects of pain.

This study shows that BDNF produced in the PB-EAC pathway is important for morphine analgesia. BDNF deletion in PB neurons leads to a significant decrease in c-Fos response to morphine in the CeL and BSTL. This deletion does not alter baseline nociceptive responses, but affects the analgesic properties of morphine. While further research is necessary to address the implications of supraspinal BDNF in nociception processes, this study provides candidate anatomical and

molecular substrates that are likely involved in opiate analgesia.

Acknowledgements

This work was supported by Centre National de la Recherche Scientifique (UPR3212) and University of Strasbourg. We thank Dr Maribel Rios for access to loxP-BDNF mice and Dr Eric Nestler for hosting parts of the experiments.

Statement of Interest

Dr Barrot received lecture fees from Adir and Lilly France and contract from Missions-Cadres. Drs Barrot and Yalcin reported a CNRS-filed patent for pain treatments.

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